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Terpenoids, flavonoids and caffeic acid derivatives from *Salvia viridis* L. cvar. Blue Jeans

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ABSTRACT

Three diterpenoids, 1-oxomicrostegiol (**1**), viroxocin (**2**), viridoquinone (**3**), were isolated from the roots of *Salvia viridis* L. cvar. Blue Jeans. Five known diterpenoids, microstegiol (**4**), 7α-acetoxy-14-hydroxy-8,13-abietadiene-11,12-dione (**5**; 7-*O*-acetylhorminone tautomer), 7α,14-dihydroxy-8,13-abietadiene-11,12-dione (**6;** horminone tautomer), ferruginol and salvinolonyl 12-methyl ether (**7**) were also found in the roots together with 1-docosyl ferulate (**8**), and a mixture of 2-(4′-alkoxyphenyl) ethyl alkanoates (**9**). Two lupane triterpenoids, 2α-acetoxy-lup-20(29)-en-3β-ol (**10**), and 3β-acetoxy-lup-20(29)-en-2α-ol (**11**) were found in the aerial parts together with known compounds, lup-20(29)-ene-2α,3β-diol (**12**), ursolic acid, oleanolic acid, β-sitosterol and β-sitosterol glucoside. A known phenylpropanoid, trans-verbascoside (or acteoside; **13**), was the main constituent in the polar fraction of the aerial part, and it is now reported in the genus *Salvia* for the first time. Other polyphenolic compounds were cis-verbascoside (**14**), leucosceptoside A (**15**), martynoside (**16**), caffeic acid, 6-*O*-caffeoyl-glucose (**18**), rosmarinic acid, salidroside, luteolin-7-*O*-α-rhamnopyranosyl-(1 → 6)-β-galactopyranoside, luteolin-7-*O*-β-galactopyranoside, luteolin-7-*O*-α-rhamnopyranosyl-(1 → 6)-β-glucopyranoside, luteolin-7-*O*-β-glucopyranoside, and apigenin-7-*O*-β-glucopyranoside. The structures were determined by 1D-, 2D-NMR and HR-ESI-MS techniques. Compounds **6**, **10**, ferruginol, ursolic acid and oleanolic acid exhibited antibacterial activity against *Enterococcus faecalis* (ATCC 775) with MIC 50 μM, 25 μM, 50 μM, 12.5 μM, 12.5 μM respectively. Ferruginol, ursolic acid and oleanolic acid were also active against *Staphylococcus aureus* (ATCC 6571), and *Bacillus cereus* (ATCC 2599) with MIC 12.5–50 μM. **4** was also active against *S. aureus* (ATCC 6571) with MIC 50 μM. These values are consistent with previous studies on the antimicrobial activity of *Salvia* diterpenoids.

1. Introduction

Various species of *Salvia* of the family Lamiaceae have long been used as traditional medicines and for culinary purposes. There are over 900 species of *Salvia* worldwide, about half of them in Central and South America (ca. 500 species) while the others are in Central Asia and the Mediterranean region (ca. 250 species), Eastern Asia (ca. 90 species), and Southern Africa (ca. 30 species) (Walker et al., 2004). Phytochemical studies on *Salvia* species have been extensively carried out, and their main chemical constituents can be classified as polyphenols and terpenoids. Various caffeic acid derivatives have been found in this genus (Lu and Foo, 2002). The major compounds of the aerial parts are flavonoids, triterpenoids and volatile substances, mainly monoterpenes, whereas diterpenoids are commonly found in the roots (Topcu, 2006).

Salvia viridis L. (Synonym *Salvia horminum* L.), or Red Topped Sage, is a perennial, annual, or biennial herb with erect stems up to 50 cm, 4–8 flowers in axillary verticillasters with various colored bracts (Hedge, 1972). Various cultivars with colored bracts are grown as garden ornamentals. It has been used in traditional medicine, for example, an infusion of leaves as a gargle for sore gums, and has also been employed to increase the quality of liquor by putting leaves and seeds into the fermentation tank (Dweck, 2000). Previous phytochemical reports on *S. viridis* L. and its synonym, *S. horminum* reported the presence of triterpenoids (Ulubelen and Brieskorn, 1975; Ulubelen et al., 1977), volatile oils (Kokkalou et al., 1982) and flavonoids (Kokkalou and Kapetanidis, 1988) in the aerial parts and diterpenoids in the roots (Ulubelen et al., 2000). These reports focussed on particular chemical groups in either roots or aerial parts of plants collected in the wild. The

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present study aimed to investigate the constituents of both the roots and aerial parts of the cultivar "Blue Jeans" to discover their respective secondary metabolite profiles across a range of biosynthetic pathways. The antibacterial activity of several of these chemical constituents was examined to complement previous reports (Moujir et al., 1993; Ulubelen et al., 2000).

2. Results and discussion

In the present study, roots and aerial parts of *S. viridis* L. were extracted and fractionated separately and gave a total of 31 compounds including those shown in Fig. 1. The non-polar fractions derived from roots of *S. viridis* L. yielded a series of abietane diterpenoids including three novel compounds **1**, **2**, **3**, and microstegiol (**4**), 7α-acetoxy-14-hydroxy-8,13-abietadiene-11,12-dione (**5**; 7-*O*-acetylhorminone tautomer), 7α,14-dihydroxy-8,13-abietadiene-11,12-dione (**6;** horminone tautomer), ferruginol and salvinolonyl 12-methyl ether (**7**) that had been previously reported.

The ^{1}H NMR spectra of all the diterpenoids showed signals for two overlapped methyl doublets coupled to a methine septet typical of an isopropyl group, and five methyl signals in all which suggests an abietane or rearranged abietane skeleton.

High resolution MS of compound **4** suggested a molecular formula of $C_{20}H_{26}O_2$ and its ^{1}H and ^{13}C NMR spectra (Table 1) were identical to those reported for microstegiol (Ulubelen et al., 1992).

Compound **1** ($C_{20}H_{24}O_3$) had similar ^{1}H and ^{13}C NMR spectra to microstegiol except for the presence of an additional ketone signal at δ 207.21 in the ^{13}C NMR spectrum and one fewer methylene signals in the DEPT spectra. The signal at δ 207.21 showed HMBC correlation (Fig. 2) to a proton at δ 3.11 (H-2 axial) while H-2 equatorial at δ 2.30 showed a long range HMBC correlation to an aromatic carbon signal at δ 138.14 (C-9). The presence of a ketone group at C-1 caused the adjacent aromatic carbon atom (C-10) to resonate at somewhat higher field (δ 137.56) than the equivalent carbon atom in microstegiol (δ 143.25). Using data tables published in the spectroscopic interpretation handbook (Williams and Fleming, 2008), a predicted chemical shift was calculated for C-10 in microstegiol as δ 141.6 and for 1-oxomicrostegiol as δ 135.2. When compared with the actual values of δ 143.3 and δ 137.6 respectively, both these values are well within the margin of error to be expected from the application of these empirical tables to a tetra-substituted aromatic compound. Moreover, the difference between the two pairs of values is 6.4 ppm for the calculated values and 5.7 ppm for the measured values. Other protons and carbon atoms were assigned by HMBC and NOESY correlations

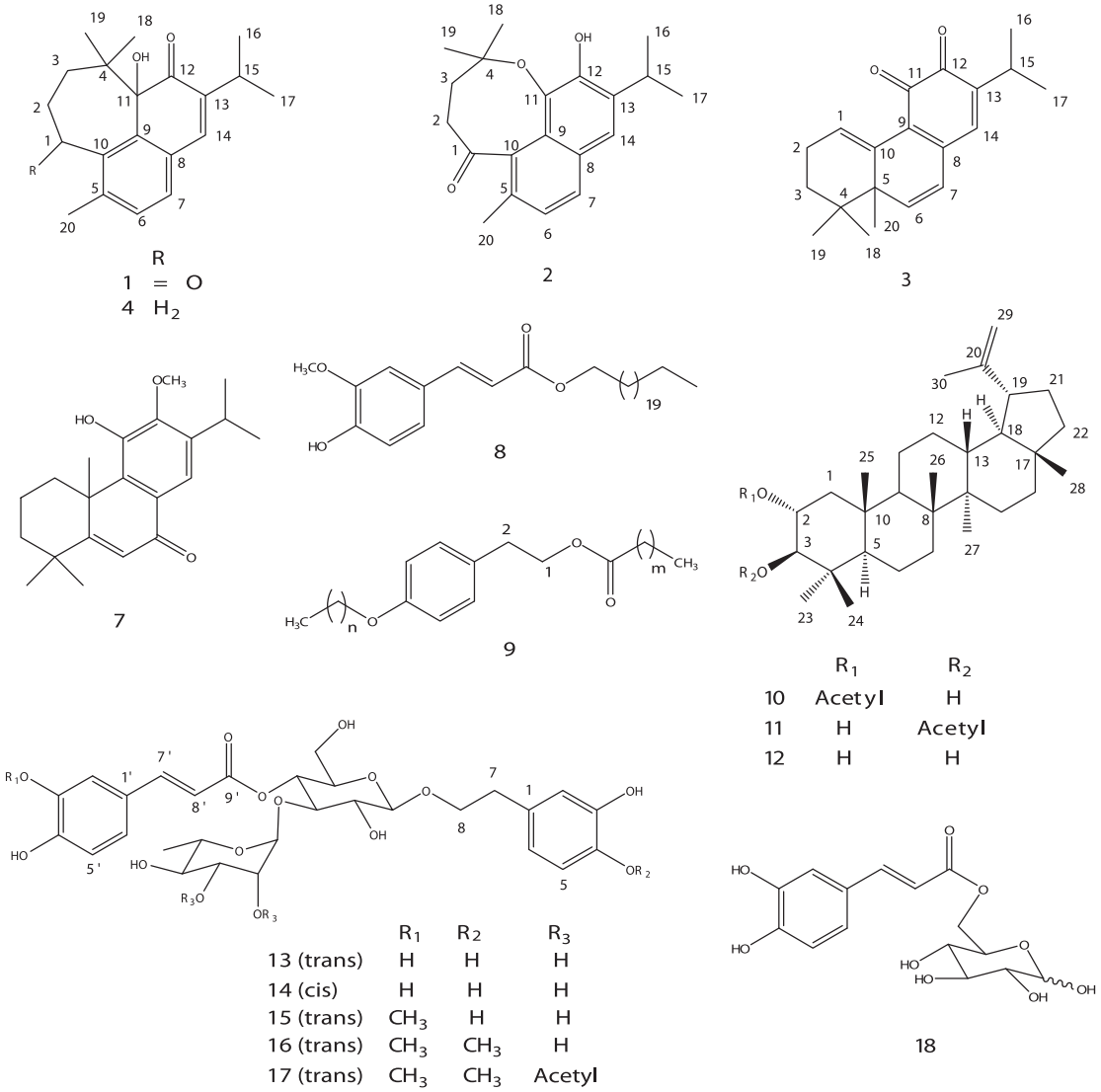

Fig. 1. Compounds isolated from *S. viridis* L. cvar. Blue Jeans (Diterpenoid numbering follows a biogenetic terpenoid system rather than the IUPAC system).

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Table 1
NMR data of rearranged diterpenoids **1**, **2** and **4** in $CDCl_3$ (500 MHz for ^1H; 125 MHz for ^{13}C; δ in ppm; J in Hz).

Position	**1**		**2**		**4**	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	207.21	-	210.85	-	27.02	2.79 (equ.) (ddd,14, 6, 3) 3.59 (ax.) (ddd,14, 12, 3)
2	38.23	3.11 (ax.) (ddd,14, 11.5, 3) 2.30[1] (equ.) (ddd, 14, 5, 3)	41.20	3.02 (ax.) (ddd,14, 10.5, 4) 2.60 (equ.) (ddd,14, 4, 4)	23.50	1.41 (m) 1.78 (m)
3	37.47	1.42 (equ.) (ddd, 14, 5, 3) 1.54[1]	35.11	1.60[1] 2.17 (ax.) (ddd,14, 10.5, 4)	42.90	2.35[1] (m) 1.24[1] (m)
4	40.94	-	84.28	-	39.02	-
5	136.88	-	130.38	-	137.37	-
6	131.18	7.22 (d, 7.5)	126.27	7.11 (d, 8)	130.09	7.05 (d, 7.5)
7	129.64	7.20 (d, 7.5)	128.15	7.61 (d, 8)	126.66	6.88 (d, 7.5)
8	127.63	-	126.77[b]	-	129.03	-
9	138.14	-	126.97 [b]	-	139.35	-
10	137.56	-	132.20 [c]	-	143.25	-
11	81.46	3.88 (OH, s)	132.08 [c]	-	84.37	4.50 (OH, s)
12	203.53	-	147.38	5.90 (OH,s)(0.5H) 5.91 (OH,s)(0.5H)	206.12	-
13	141.79	-	136.58	-	141.01[e]	-
14	139.08	7.07 (s)	121.17	7.41 (s)	140.94 [e]	6.95 (s)
15	26.92	2.98 (septet, 7)	27.83	3.34 (septet, 7)	26.82	3.00 (septet, 6)
16	21.86[a]	1.16 (3H, d, 7)	22.34 [d]	1.33 (3H, d, 7)	22.07 [f]	1.14 (3H, d, 7)
17	21.51[a]	1.18 (3H, d, 7)	22.30 [d]	1.28 (3H, d, 7)	21.09 [f]	1.19 (3H, d, 7)
18	17.38	1.05 (3H, s)	26.02	1.23 (3H,s)	21.66	0.79 (3H, s)
19	26.88	0.80 (3H, s)	26.25	1.62 (3H,s)	28.90	0.78 (3H, s)
20	19.85	2.34 (3H, s)	18.89	2.33 (3H,s)	21.37	2.31 (3H, s)

[1] Overlapped signal a, b, c, d, e, f Interchangeable.

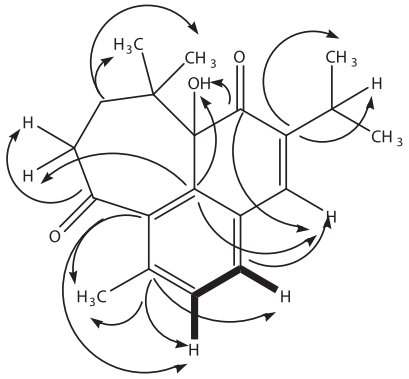

Fig. 2. Selected HMBC (arrow) and H-H COSY (bold line) correlations of compound **1** (1-oxomicrostegiol).

as depicted in Figs. 2 and 3. Therefore, compound **1** was identified as 1-oxomicrostegiol. An isomer of this compound, 3-oxomicrostegiol, was reported from the roots of *Taiwania cryptomerioides*, family Taxodiaceae (Chyu et al., 2005).

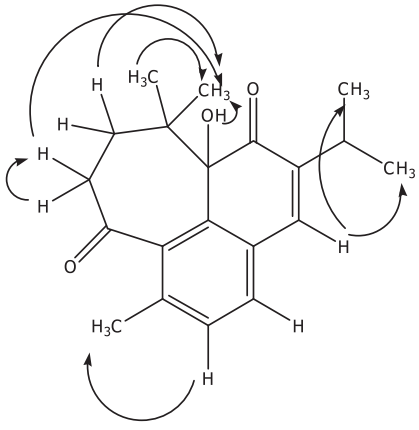

Fig. 3. Selected NOESY correlations of compound **1** (1-oxomicrostegiol).

Compound **2** ($C_{20}H_{24}O_3$) has the same molecular formula as compound **1** but there was only one ketone carbon at δ 210.85, the signal of C-quaternary (C-4) around δ 40 was missing whilst a new quaternary signal was observed at δ 84.28. ^{13}C NMR with DEPT-90 and DEPT-135 experiments displayed 20 signals comprised of two methylene groups, four methine groups, five methyl groups, and nine quaternary carbon atoms. In all, ten signals, for three methine and seven quaternary carbon atoms occurred in the aromatic region of the ^{13}C NMR spectrum, suggesting a possible naphthalene derivative. As in compound **1** the typical signals of an isopropyl side chain and the three aromatic protons at δ 7.11 (H-6), δ 7.61 (H-7), and δ 7.41 (H-14) pointed to an abietane type diterpenoid skeleton. The ketone carbon at δ 210.85 correlated to a methylene proton at δ 2.60 by HMBC (Fig. 4) suggesting that this was part of the aliphatic ring. This methylene proton was one of four coupled together, indicating two adjacent methylene groups. All the above suggested a rearranged abietane diterpenoid containing a substituted naphthalene ring system within the structure. The quaternary carbon atom at δ 84.28 was assigned to the C-4 position because it had HMBC correlation to the geminal methyl groups at δ 1.62 (H-19), and δ 1.23 (H-18). The chemical

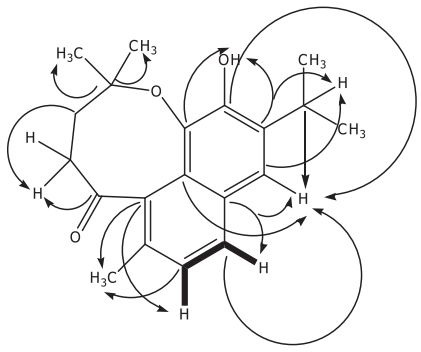

Fig. 4. Selected HMBC (arrow) and H-H COSY (bold line) correlations of compound **2** (viroxocin).

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shift indicates attachment to an electronegative atom. Other proton and carbon assignments were made using HMBC and COSY correlations as depicted in Fig. 4. Thus, compound **2** was identified as 3,4-dihydro-11-hydroxy-10-(1-methylethyl)-2,2,6-trimethyl-naphtha[1,8-bc]oxocin-5(2H)-one), a novel compound for which we propose the trivial name viroxocin. This is only the third report of a natural compound of this skeletal type. Previously, rabdosin D and 10-isopropyl-2,2,6-trimethyl-2,3,4,5- tetrahydro-naphtha-[1,8-bc]oxocin-5,11-diol were reported from whole plant of *Rabdosia iophanthides* (Buch.-Ham. Ex. D. Don) Hara. var. *gerardiana* (Benth.) Hara., family Lamiaceae (Li et al., 2010), and from the roots and rhizomes of *Nardostachys chinensis* Batalin, family Valerianaceae (Zhang et al., 2005) respectively, and a compound named as 4,11-epoxy-12-methoxy-4,5-seco-20(10–5)-*abeo*-abieta-5(10),6,8,11,13-pentaene was reported as a semisynthetic product from the acid cyclization of aethiopinone (Cuadrado et al., 1992). The ^{1}H and ^{13}C NMR spectra of viroxocin are very similar to those previously reported for these compounds; in particular, the naphthalene core gives very similar signals in both ^{1}H and ^{13}C NMR spectra. It is notable that the chemical structure of 10-isopropyl-2,2,6-trimethyl-2,3,4,5- tetrahydronaphtha-[1,8-bc]oxocin-5,11-diol was supported by X-ray crystal data. This compound differs from viroxocin by the presence of a secondary alcohol group instead of carbonyl at C-1, whereas Rabdosin D differs only by hydroxylation at C-15. The ^{13}C and ^{1}H NMR data for the naphthalene ring system (carbon atoms 5 to 14 inclusive) are almost identical for all three compounds, whilst viroxocin differs from the nardostachys compound at positions C-1, C-2 and C-3 and from Rabdosin D at positions C-15, C-16 and C-17 (a terpenoid numbering system is applied for this comparison).

A further point of interest in the ^{1}H NMR spectrum of viroxocin is the hydroxyl group which shows two sharp singlets of equal intensity, both integrating to 0.5 protons at δ 5.90 and δ 5.91; both were exchanged with D_2O. This is unlikely to be the result of coupling since there are no protons with which the hydroxyl proton might couple, and no indication of coupling in the COSY spectrum. We hypothesise that the two signals reflect two different proton environments resulting from hindered rotation of the C-O bond at position 12. This is likely to be hindered by both the isopropyl substituent at position 13 which itself shows hindered rotation and by hydrogen bonding to the fixed ether oxygen at position 11. Thus, the hydroxyl proton would be displaced to one side or the other of the plane of the naphthalene ring. The eight-membered aliphatic A ring is not planar, and models show that the oxygen atom of the keto group on this ring is rotated out of the plane of the naphthalene ring. Thus, the anisotropic effects of the keto group are likely to create different magnetic environments on either side of the plane of the naphthalene ring system.

HR-ESI-MS of compound **3** suggested the molecular formula $C_{20}H_{24}O_2$. ^{13}C NMR (Table 2) of compound **3** displayed 20 signals, and DEPT-135 showed two methylene groups, eight quaternary carbon atoms, and ten signals of methine and methyl groups. Four ^{1}H signals resonated in the aromatic and alkene region. Two doublet signals, δ 5.97 (H-7) and δ 6.46 (H-6) were coupled to each other with coupling constant 9.5 Hz suggesting a cis double bond possibly within a ring. NOESY (Fig. 5) correlated the signal at δ 5.97 (H-7) to a singlet signal at δ 6.58 (H-14) in the adjacent ring. There were two quaternary carbon signals at δ 180.54, and δ 180.48. These shielded carbonyl groups suggested a quinone rather than two isolated ketone carbon atoms as observed in compound **1**. Both also had HMBC correlations to the ^{1}H signal δ 6.58 (H-14). HMBC (Fig. 6) also correlated C-14 at δ 137.02 to the septet signal at δ 2.95 (H-15). The remaining alkene ^{1}H signal at δ 6.72 showed no correlation to the signals described above and resonated as triplet signal with coupling constant 4.5 Hz. COSY and NOESY spectra showed a strong correlation of this triplet to methylene protons at

Table 2
NMR data of compound **3** in $CDCl_3$ (500 MHz for ^{1}H; 125 MHz for ^{13}C, δ in ppm; *J* in Hz).

Position	**3**	
	δ_C	δ_H
1	134.36	6.72 (t, 4.5)
2	23.85	2.30[1] (2H, m)
3	31.84	1.26[1] (m)
		1.86 (ddd, 14, 10, 5)
4	33.11	-
5	43.37	-
6	146.57	6.46 (d, 9.5)
7	123.91	5.97 (d, 9.5)
8	137.18 [a]	-
9	125.41	-
10	131.69	-
11	180.54 [b]	-
12	180.48 [b]	-
13	146.90	-
14	137.02 [a]	6.58 (s)
15	27.18	2.95 (septet, 6)
16	21.55 [c]	1.12 (3H, d, 7)
17	21.42 [c]	1.10 (3H, d, 7)
18	24.52	1.02 (3H, s)
19	25.74	0.91 (3H, s)
20	23.45	1.08 (3H, s)

[1] Overlapped signal; a, b, c Interchangeable.

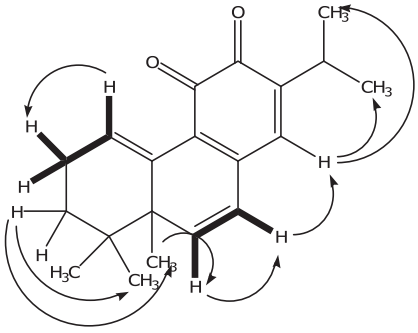

Fig. 5. Selected NOESY (arrow) and H-H COSY (bold line) correlations of compound **3** (viridoquinone.).

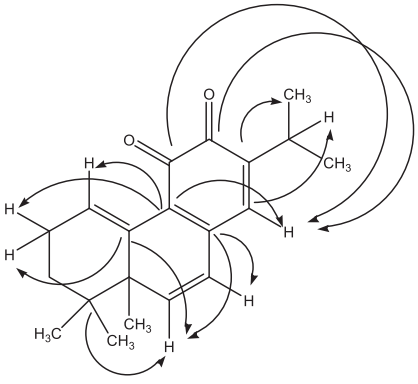

Fig. 6. Selected HMBC correlations of compound **3** (viridoquinone).

δ 2.30 supported by an HMBC correlation with the ^{13}C NMR signal at δ 23.85. Therefore, the structure is likely to be a substituted quinone with four conjugated double bonds.

Unlike the typical abietane type, a methyl singlet signal at δ 1.08 had a NOESY correlation to an alkene proton H-6 (δ 6.46). This observation suggested a rearranged diterpenoid with the methyl group C-20 at C-5 instead of C-10. Other proton and carbon atoms

were assigned using NOESY, COSY and HMBC correlations as depicted in Figs. 5 and 6. This compound was thus identified as 5-methyl-20-nor-abieta-1(10),6,8,13-tetraen-11,12-dione for which we propose the trivial name viridoquinone. This is the third report of a compound with this skeletal type. Previously, pygmaeocin B and salviskinone A were reported from the roots of *Pygmaeopremna herbacea* (Roxb.) Moldenke, family Verbenaceae (Meng and Hesse, 1990) and from the roots of *Salvia przewalskii* Maxim. (Ohsaki et al., 2011) respectively. Pygmaeocin B has the same B and C ring structure as viridoquinone and very similar ^{13}C and ^{1}H NMR signals for these atoms whereas salviskinone A has the same A and B ring structure as viridoquinone and shows very close spectroscopic similarity for this region.

Compound **5** ($C_{22}H_{30}O_5$) and compound **6** ($C_{20}H_{28}O_4$) occurred in the same fraction in column chromatography but were separated by the solubility of compound **6** in methanol. HR-ESI-MS positive ion mode of the mixture displayed two peaks at *m*/*z* 397.1974 and 355.1880 while the negative mode also showed the corresponding peaks at *m*/*z* 373.2004, and 331.1898. The 42 difference in molecular weight implied acetylation.

Compounds **5** and **6** had identical NMR spectra to the published NMR data for the known compounds 7-*O*-acetylhorminone and horminone respectively (Tezuka et al., 1998). These compounds are commonly drawn as the *p*-quinone tautomers (Fig. 7). However, in the present study compound **5** showed an HMBC correlation between the proton at H-7 (δ 5.92) and a carbon signal at δ 150.7 (C-14) which is more consistent with the *o*-quinone tautomer (Fig. 7). In addition, in compound **6** a NOESY correlation was observed between the phenolic group resonating at δ 7.20 and H-7 (δ 4.72). This also is more consistent with the *o*-quinone structure. Thus, these data would suggest that these compounds exist as tautomeric mixtures which include a significant proportion of the *o*-quinone tautomer. This has not previously been reported and is possibly only determinable by these 2D NMR spectroscopic techniques. 7-*O*-Acetylhorminone and horminone have been reported in many *Salvia* species (Oztekin et al., 2010).

The typical abietane diterpenoid, ferruginol, and microstegiol (**4**) with a rearranged abietane skeleton have previously been reported from *S. viridis* L. roots (Ulubelen et al., 2000). These authors also reported the A-seco abietane, aethiopinone, which was not found in the current study. Viroxocin has an A-seco carbon skeleton in which the A ring has reclosed by an ether linkage to form an eight-membered oxocane ring. In fact, a compound containing an oxocane ring was experimentally obtained from the reaction of aethiopinone with $BF_3.Et_2O$ in methanol for 24 h at room temperature (Cuadrado et al., 1992). An alternative route to the oxocane ring system might occur via aromatization of the microstegiol skeleton in which 1-oxomicrostegiol (**1**) would be a direct precursor for viroxocin (**2**). The microstegiol skeleton may also arise via an A-seco intermediate which could further be oxidized to **1**. These A-seco derived diterpenoids also feature the presence of the C-20 methyl group substituted at position 5 rather than

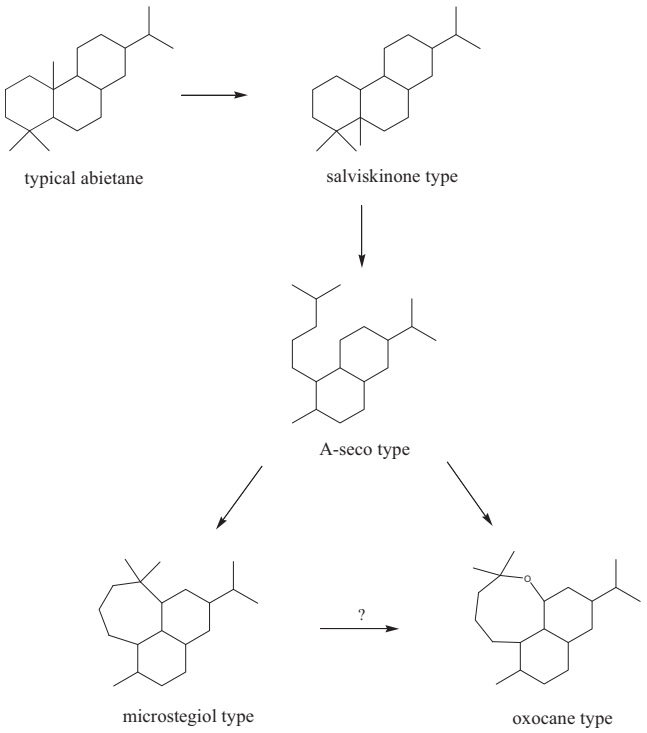

Fig. 8. Hypothetical biogenetic sequence to some rearranged abietane diterpenes in *Salvia*.

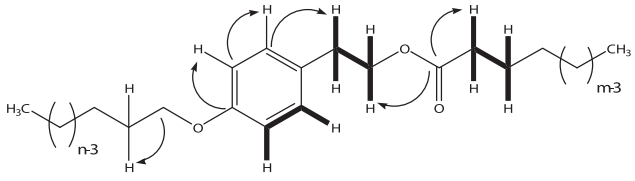

Fig. 9. Selected HMBC (arrow) and H-H COSY (bold line) correlations of compound **9**.

position 10 as in a typical abietane. Viridoquinone (**3**) may thus represent an intermediate skeleton in which the methyl shift has occurred but the A ring remains intact. A hypothetical biogenetic sequence based on these observations is shown in Fig. 8. This is supported by the proposed mechanism for the semisynthetic conversion of a typical abietane to a microstegiol derivative under mild acid conditions reported by Simoes et al. (2010).

The non-polar fractions of root also yielded a mixture of fatty esters of an aromatic ether. ^{13}C NMR and DEPT-135 of a mixture of fatty esters (**9**) displayed two signals of methine groups at δ 115.29 and δ 130.04 which had HMQC cross-peaks to a pair of

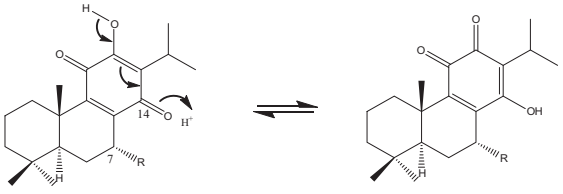

Fig. 7. Tautomers of abietane quinones with hydroxyl substitution.

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Table 3
NMR data of isolated lupane triterpenoids in $CDCl_3$ (500 MHz for ^1H; 125 MHz for ^{13}C; δ in ppm; J in Hz).

Position	**10**		**11**		**12**	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	44.0	0.90	48.1	0.90[1]	46.7	0.83[1]
		2.04[1]		2.09[1]		2.03[1]
2β	73.6	4.86 (ddd, 10, 10, 5)	67.8	3.77 (ddd, 10, 10, 5)	69.3	3.67 (ddd, 10, 9.5, 4.5)
3α	80.9	3.15 (d, 10)	85.0	4.46 (d, 10)	83.9	2.97 (d, 9.5)
4	39.8	-	39.3	-	38.6	-
5	55.2	0.76[1]	55.3	0.88[1]	55.4	0.79[1]
6	18.2	1.40[1]	18.3	1.40[1]	18.3	1.40[1]
		1.53[1]		1.50[1]		1.52[1]
7	34.1	1.43[1] (2H)	34.1	1.40[1] (2H)	34.2	1.39[1] (2H)
8	40.9	-	40.9	-	40.9	-
9	50.4	1.31[1]	50.3	1.34[1]	50.4	1.32[1]
10	38.5	-	38.4	-	39.2	-
11	21.1	1.25[1]	21.1	1.26[1]	21.0	1.26[1]
		1.40[1]		1.43[1]		1.43[1]
12	25.0	1.07[1]	25.0	1.08[1]	25.0	1.08[1]
		1.66[1]		1.68[1]		1.67[1]
13	38.0	1.64[1]	37.9	1.65[1]	38.0	1.65[1]
14	42.9	-	42.9	-	42.9	-
15	27.4	0.98[1]	27.4	0.98[1]	27.4	1.05[1]
		1.66[1]		1.68[1]		1.74[1]
16	35.6	1.37[1]	35.5	1.38[1]	35.6	1.38[1]
		1.47[1]		1.46[1]		1.47[1]
17	43.0	-	43.0	-	43.0	-
18	48.3	1.34[1]	48.2	1.36[1]	48.3	1.35[1]
19	47.9	2.37 (ddd, 11, 11, 6)	48.0	2.37 (ddd, 11, 11, 6)	48.0	2.36 (ddd, 11, 11, 6)
20	150.8	-	150.8	-	150.9	-
21	29.7	1.38[1]	29.8	1.32[1]	29.8	1.32[1]
		1.92 (m)		1.90 (m)		1.92 (m)
22	40.0	1.19[1]	40.0	1.20[1]	40.0	1.20[1]
		1.38[1]		1.40[1]		1.38[1]
23	28.4	1.03 (3H, s)	28.4	0.87 (3H, s)	28.5	1.01 (3H, s)
24	16.4	0.84 (3H, s)	17.4	0.85 (3H, s)	16.5	0.80 (3H, s)
25	17.1	0.94 (3H, s)	17.3	0.91 (3H, s)	17.4	0.90 (3H, s)
26	16.0	1.02 (3H, s)	16.0	1.02 (3H, s)	16.0	1.02 (3H, s)
27	14.5	0.93 (3H, s)	14.5	0.94 (3H, s)	14.5	0.94 (3H, s)
28	18.0	0.78 (3H, s)	18.0	0.78 (3H, s)	18.0	0.78 (3H, s)
29	109.4	4.55 (d, 2)	109.4	4.57 (d, 2.5)	109.4	4.57 (d, 2)
		4.67 (d, 2)		4.68 (d, 2.5)		4.69 (d, 2)
30	19.3	1.65 (3H, s)	19.3	1.68 (3H, s)	19.3	1.67 (3H, s)
Acetyl group	21.3	2.05 (3H, s)	21.0	2.13 (3H, s)	-	-
	171.6	-	172.4	-	-	-

[1] Overlapped signal.

two proton doublets at δ 6.75 and δ 7.07 coupled to each other with ^3J value 8.5 Hz typical of a para-disubstituted aromatic compound. Two methylene triplets at δ 2.85 and δ 4.23 were coupled to each other with ^3J value 7 Hz. The triplet signal at δ 2.85 had a HMBC cross-peak (Fig. 9) to the aromatic carbon atom at δ 130.04 while the other triplet at δ 4.23 had HMBC correlation to the only carbonyl carbon atom at δ 173.86. These observations suggest the attachment of an acyl ester to the side chain of a para-substituted phenylethyl moiety.

The carbonyl signal at δ 173.86 (C-α-alkanoate) also had a HMBC correlation to a triplet signal of methylene protons at δ 2.27. This triplet was correlated to a broad alkane signal at δ 1.56 which had further correlations to another broad and intense alkane signal at δ 1.25.

A triplet signal at δ 3.64 showed no correlation to the other triplets but had COSY correlation to the broad singlet of alkane signals at δ 1.56. The downfield chemical shift suggested attachment to an electronegative atom and this was considered to be an oxygen atom directly attached to the aromatic ring and responsible for the shielding of the aromatic protons. The integration of an upfield triplet at δ 0.88 was shown as two methyl groups (6H). These observations suggested two alkyl chains, one substituted via an ether link to the aromatic ring and the other as part of an alkanoate acyl moiety. Thus, this compound was identified as a mixture of 2-(4'-alkoxyphenyl) ethyl alkanoates (**9**) established by NMR data; where m and n are the number of methylene groups of each chain. GC-CI-MS showed that m + n = 19 for $C_{30}H_{52}O_3$, 21 for $C_{32}H_{56}O_3$ and 23 for $C_{34}H_{60}O_3$. However, the exact length of each alkyl chain could not be determined. A related fatty acid ester, 2-(4'-pentoxyphenyl) ethyl stearate, was reported from leaves and stems of *Stemodia foliosa* Benth (Scrophulariaceae) (Silva et al., 2002). In addition, a phenolic ester, *p*-hydroxyphenylethyl docosanate was also found in the aerial parts of *Salvia cedronella* Boiss. (Yesilyurt et al., 2008). NMR data of this compound were similar to those in the literature.

In contrast to the root, the non-polar fractions of the aerial parts yielded a series of triterpenoids with lupane, ursane and oleanane skeletons.

The molecular formula of compound **12** ($C_{30}H_{50}O_2$) suggests a triterpenoid skeleton with an additional oxygen function. Comparison of the ^{13}C and ^1H NMR data (Table 3) with published values identified compound **12** as lup-(20)29-ene-2α,3β-diol, previously isolated from this species by Ulubelen et al. (1977). A methine doublet (^3J = 9.5 Hz) at δ 2.97 (H-3) coupled to a d.d.d. at δ 3.67 (H-2) confirmed that the extra hydroxyl group is at position 2. The coupling constant is consistent with diaxial coupling and thus the hydroxyl groups are both equatorial.

Compounds **10** and **11** both have the molecular formula $C_{32}H_{52}O_3$ and the NMR data for both compounds (Table 3) clearly show that they are acetylated derivatives of **12.** Both compounds

Table 4
NMR data of trans- and cis-verbascoside in CD_3OD (400 MHz for ^1H; 100 MHz for ^{13}C; δ in ppm; J in Hz).

Moiety	Position	Trans-verbascoside (**13**)		Cis-verbascoside (**14**)	
		δ_C	δ_H	δ_C	δ_H
Dihydroxy phenyl ethyl	1	131.4	-	131.4	-
	2	117.1	6.69 (d, 1.6)	116.3	6.69 (d, 2)
	3	146.1[a]	-	146.1[c]	-
	4	144.6[a]	-	144.6[c]	-
	5	116.3	6.68 (d, 8)	116.5	6.73 (d, 8.2)
	6	121.2	6.56 (dd, 8, 1.6)	121.2	6.55 (dd, 8.2, 2)
	7	36.5	2.78 (2H, m)	36.6	2.77 (2H, m)
	8	72.2	3.70 (m)	73.9	3.70 (m)
			4.05 (m)		4.02 (m)
Caffeic acid	1'	127.6	-	128.0	-
	2'	115.2	7.05 (d, 2)	119.0	7.52 (d, 1.5)
	3'	146.8[b]	-	146.8[d]	-
	4'	149.7[b]	-	149.7[d]	-
	5'	116.5	6.78 (d, 8.2)	115.2	6.77 (d 8.2)
	6'	123.2	6.95 (dd, 8.2, 2)	125.9	7.09 (dd, 8.2, 2)
	7'	148.0	7.60 (d, 16)	148.6	6.86 (d, 12.8)
	8'	114.7	6.29 (d, 16)	114.7	5.75 (d, 12.8)
	9'	168.3	-	168.3	-
Glucose	1 g	104.2	4.38 (d, 7.8)	104.1	4.34 (d, 7.8)
	2 g	76.2	3.38 (t, 9)	76.1	3.35 (t, 8)
	3 g	81.6	3.81 (t, 9)	81.9	3.76 (t, 9)
	4 g	70.5	4.91 (t, 9)	70.6	4.92[1]
	5 g	76.0	3.53[1]	76.0	3.53[1]
	6 g	60.9	3.49[1]	62.3	3.49[1]
			3.64[1]		3.64[1]
Rhamnose	1r	103.0	5.18 (s)	103.1	5.16 (s)
	2r	72.3	3.91 (d, 1.6)	72.3	3.92 (br s)
	3r	72.0	3.58[1]	72.0	3.58[1]
	4r	73.8	3.29[1] (m)	73.9	3.32[1] (m)
	5r	70.4	3.56[1]	70.4	3.56[1]
	6r	18.4	1.09 (3H, d, 6.2)	18.2	1.16 (3H, d, 6.2)

[1] Overlapped signal; a, b, c, d Interchangeable.

show a carbonyl quaternary signal at about δ 172 and a methyl signal at about δ 21. The latter signal correlates by HSQC with a ^1H NMR signal at about δ 2.1. Thus both compounds carry a single acetyl group. Compound **10** shows a methine doublet signal at δ 3.15 (H-3) coupled to a deshielded d.d.d. at δ 4.86 (H-2) whereas in compound **11**, the H-3 doublet is deshielded to δ 4.46 and the H-2 d.d.d. is at δ 3.77. Both compounds show the same configuration at these positions as compound **12**. Therefore, compound **10** was identified as 2α-acetoxy-lup-(20)29-en-3β-ol. This is a novel natural product though it has previously been synthesized from compound **12** (Kumar and Seshadri, 1976). Compound **11** is a novel isomer, 3β-acetoxy-lup-(20)29-en-2α-ol. An epimer of compound **11**, 3α-acetoxy-lup-(20)29-en-2α-ol, was recently reported in *Salvia trijuga* Diels (Pan et al., 2010).

The characteristic components of the polar fractions from the aerial parts were the caffeic acid derivatives, trans-verbascoside (**13**) (Nishimura et al., 1991), cis-verbascoside (**14**) (Nishimura et al., 1991), leucosceptoside A (**15**) (Kim et al., 2001), martynoside (**16**) (Kim et al., 2001), caffeic acid (compared to a standard), 6-*O*-caffeoyl-glucose (**18**) (Gao et al., 1999), rosmarinic acid (compared to a standard) and salidroside (Yu et al., 2007).

The NMR spectra of trans- and cis-verbascoside were almost identical except for the alkene protons of the caffeoyl moiety as shown in Table 4. In trans-verbascoside these resonated at δ 7.60 and δ 6.29 with J = 16 Hz whereas in the cis isomer these protons resonated at δ 6.86 and δ 5.75 respectively with J = 12.8 Hz. This marked upfield shift suggests that the bulky glycoside ester group prevents the cis isomer adopting a conformation in which the caffeoyl alkene group is periplanar with the aromatic ring. Thus the alkene π electrons are unable to delocalize into the aromatic system. This contrasts with the coumarin, esculin, in which the cis alkene group is locked into a periplanar position by the lactone ring. In this case the chemical shifts of the corresponding alkene protons are δ 7.84 and δ 6.21 (Bayoumi et al., 2010).

Nishimura et al. (1991) suggested that cis-verbascoside was converted to its trans-isomer in daylight, though they presented no experimental evidence. In the present study, irradiation of a sample containing over 98% trans-verbascoside with UVA to reproduce the ultraviolet component of daylight increased the proportion of the cis isomer to 30%. This is consistent with studies on a series of chlorogenic acids (quinic acid esters of caffeic acid) in which irradiation of the trans-isomer with UV light at 254 nm gave an increase in the proportion of cis isomer (Parveen et al., 2011). It is notable that in the present study, verbascoside was the only one of the caffeic acid esters for which the cis-isomer was observed. This suggests that cis-verbascoside was not an artifact of the isolation process. It is generally accepted that the enzyme Phenylalanine-Ammonia Lyase (PAL) yields the trans-isomer of cinnamic acid. Whilst it is clearly possible that cis derivatives may arise by photoisomerisation, recent studies have demonstrated that in the biosynthesis of coumarins in Cassava, trans → cis isomerisation of a cinnamic acid intermediate involves a non-light dependent, enzyme-catalysed process (Bayoumi et al., 2008).

The other known compounds were determined by NMR and HR-ESI-MS, and by comparison with previous reports. Microstegiol (**4**) was isolated from the aerial part of *Salvia microstegia* Boiss. Et Bal. (Ulubelen et al., 1992), and also in the roots of *Salvia montbretii* Benth. (Topcu and Ulubelen, 1996), and *S. viridis* L. (Ulubelen et al., 2000). Ferruginol has previously been isolated from the roots of plants in the genus *Salvia*, for instance *S. viridis* L. (Ulubelen et al., 2000), *Salvia miltiorrhiza* Bunge (Lee et al., 2005), *Salvia cilicica* Boiss and Kotschy (Tan et al., 2002), *Salvia deserta* Schang

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Table 5
MIC (μM) of isolated compounds.

Compound	*Staphylococcus aureus* (ATCC 6571)	*Bacillus cereus* (ATCC 2599)	*Enterococcus faecalis* (ATCC 775)
Compound **1**	200	200	100
Compound **2**	100	200	100
Compound **3**	100	100	100
Microstegiol **4**	50	100	100
7-*O*-Acetylhorminone tautomer **5**	100	200	100
Horminone tautomer **6**	100	100	50
Ferruginol	25	50	25
Salvinolonyl-12-methyl ether **7**	200	200	200
1-Docosyl ferulate **8**	200	200	>200
Compound **10**	100	100	50
Compound **11**	100	100	100
Lup-20(29)-ene-2α-3β-diol **12**	100	100	100
Ursolic acid	25	50	12.5
Oleanolic acid	50	12.5	12.5
β-Sitosterol	100	50	100
β-Sitosterol glucoside	>200	100	>200
Verbascoside **13**	100	100	100
Caffeic acid	100	100	100
Rosmarinic acid	200	100	200
Luteolin-7-glucoside	100	100	200
Chloramphenicol	7.8	1	2

(Tezuka et al., 1998). Salvinolonyl 12-methyl ether (**7**) was previously reported from *S. montbretii* Benth. (Gil et al., 1994). A long chain alkyl ester, 1-docosyl ferulate (**8**), was previously reported from the stem bark of *Pavetta owariensis* P. Beauv. (Rubiaceae) (Balde et al., 1991) and the whole plant of *Teucrium divaricatum* subsp. *villosum* (Celak) Rech. fil. (Lamiaceae) (Ulubelen et al., 1994). The polar fraction of the root extract contained 2'', 3''-Di-*O*-acetyl martynoside (**17**) (Abe et al., 2002). Of the aerial constituents, Lup-(20)29-ene-2α,3β-diol (**12**) has been previously reported in *S. viridis* L. (Ulubelen et al., 1977). Other triterpenoids were ursolic acid (Seebacher et al., 2003), oleanolic acid (Seebacher et al., 2003), β-sitosterol (Kojima et al., 1990) and β-sitosterol glucoside (Kojima et al., 1990). The polar fractions of the aerial parts also yielded a series of flavonoid glycosides; luteolin-7-rutinoside (Siciliano et al., 2004), luteolin-7-glucoside (Siciliano et al., 2004), apigenin-7-glucoside (Bennini et al., 1992). Luteolin-7-*O*-α-rhamnopyranosyl-(1 → 6)-β-galactopyranoside was first reported from *Pteris cretica* (Imperato, 1994). Luteolin-7-*O*-β-galactopyranoside was reported in *Verbena officinalis* L. and *V. supina* L. (Kawashty and El-Garf, 2000).

2.1. Antibacterial activity

Table 5 shows the antibacterial activity of some of the isolated compounds. These moderate levels of activity lie in a similar range to those previously reported for *Salvia* diterpenoids using slightly different panels of bacteria (Moujir et al. 1993; Ulubelen et al., 2000). Compound **4** was active against *Staphylococcus aureus* (ATCC 6571) with MIC 50 μM while the oxo-substitution at C-1 (compounds **1**, **2**) reduced the activity. Moujir et al. (1993) reported the structure activity relationship of abietane diterpenes from *Salvia* against Gram positive bacteria. They suggested that compounds containing a catechol or an ortho- quinone group at C-11 and C-12 showed enhanced the antibacterial activity while substitution at C-6 or C-7 reduced it. Among three *o*-quinone compounds tested in the present study, **6** showed antibacterial activity against *Enterococcus faecalis* (ATCC 775) with MIC 50 μM while **3** and **5** were less active. Moreover, ferruginol, which lacks the catechol substitution, proved to be the most active of the diterpenoids in the present study. These results provide only partial support for previous studies and suggest that other structural features may influence activity.

In the present study, ursolic acid and oleanolic acid exhibited appreciable antibacterial activity against *E. faecalis* (ATCC 775) with MIC 12.5 μM. Oleanolic acid was superior to ursolic acid against *Bacillus cereus* (ATCC 2599). Acetylation at C-2 in the lupene diol skeleton increased the activity as a new lupane triterpenoid **10** was active against *E. faecalis* (ATCC 775) with MIC 50 μM when compared to **11** and **12** which were active at 100 μM. All crude fractions from the aerial parts showed no activity against Gram negative bacteria, *Klebsiella pneumoniae* (ATCC 9633), *Proteus vulgaris* (ATCC 4636), and *Escherichia coli* (ATCC 86) by TLC agar overlay assay (data not shown). DMSO toxicity was observed at a concentration of 10%.

3. Conclusions

In this study, eight diterpenoids, a fatty ester (**8**), two phenylpropanoids (**13, 17**), and rosmarinic acid were found in the roots of *S. viridis* cv. Blue Jeans as well as a mixture of 2-(4'-alkoxyphenyl) ethyl alkanoates (**9**), ursolic acid, oleanolic acid and β-sitosterol. Compounds **1**, **2** and **3** are novel diterpenoids with rearranged abietane skeletons for which we propose the trivial names 1-oxomicrostegiol, viroxocin, and viridoquinone respectively. Although the abietane skeleton is widespread in plants and numerous substitution variants are known, the distribution and substitution patterns of rearranged diterpenoids have not been extensively reported.

Twenty compounds were isolated from the aerial parts including two new natural lupane triterpenoids, compounds **10** and **11**. Previous reports of triterpenoids in this species include lup-(20)29-ene-2α,3β-diol (**12**) (Ulubelen et al., 1977), ursolic acid, oleanolic acid (Ulubelen and Brieskorn, 1975). Caffeic acid and the flavonoids, luteolin-7-*O*-α-rhamnopyranosyl-(1 → 6)-β-glucopyranoside, luteolin-7-*O*-β-glucopyranoside, and apigenin-7-*O*-β-glucopyranoside were reported by Kokkalou and Kapetanidis (1988). In addition to these, two luteolin glycosides containing galactose are reported in the present study.

Although it has not previously been reported from *Salvia,* in the present study, verbascoside (**13**) (acteoside) was the major compound isolated from the aerial parts of the plant together with lesser amounts of the methylated analogs, **14**, **15** and **16.** The caffeic acid part of this molecule is derived from phenylalanine via cinnamic acid whereas the phenylethyl moiety is derived from

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tyrosine. (Ellis, 1983; Saimaru and Orihara, 2010). Salidroside, a possible intermediate in the phenylethyl pathway, was also found as a minor component of *S. viridis* in the present study.

These profiles of secondary metabolites originating from several different biosynthetic pathways may provide interesting insights into the relationships between the living plant and its total complement of chemical constituents.

4. Experimental

4.1. General experimental procedures

NMR experiments were carried out on Bruker spectrometers (400 MHz for ^{1}H and 100 MHz for ^{13}C; 500 MHz for ^{1}H and 125 MHz for ^{13}C). Chemical shifts (δ) and coupling constants (J) were recorded in parts per million (ppm), and Hertz (Hz) respectively. Mass spectra were performed on a Bruker micrOTOF mass spectrometer using electrospray ionization (HR-ESI-MS) at the Department of Pharmacy and Pharmacology, University of Bath and GC-CI-MS analysis at the EPSRC National Mass Spectrometry Service Center, School of Medicine, University of Swansea. The HPLC instruments used were: Jasco PU-980 pump, Jasco UV-975 detector, Goerz Metrawatt Servogor 120 recorder, and a commercial reversed-phase HPLC column, Phenomenex Luna PFP 250 × 10 mm. A detection wavelength of 330 nm, and flow rate of 5 ml/min, were used for all crude samples. All mobile phase solvents were HPLC grade, filtered through a 0.45 μm nylon filter, and sonicated 30 min by a Decon ultrasonicator before being used. The sample was dissolved in methanol and also filtered through 0.45 μm nylon Acrodisc prior to loading into the injection loop. For semi-preparative HPLC, the sample was repeatedly injected and the eluent corresponding to each peak on the recorder was collected manually. TLC plates, aluminum-backed sheets coated with silica GF_{254} 60 in 0.25 mm thickness were purchased from Merck. The mobile phase used to develop the TLC plates was ethyl acetate: methanol:formic acid:water (50:3:3:6) unless stated otherwise. The developed TLC plates were observed in visible light and under UV light at 254 nm and 365 nm, and after spraying with anisaldehyde-sulfuric acid reagent. Caffeic acid and rosmarinic acid were purchased from Sigma and Aldrich respectively.

4.2. Plant material

S. viridis L. cv. Blue Jeans was grown in Bath from commercially available seed purchased from Thompson and Morgan, and harvested in September. Aerial parts and roots were separated, air-dried at room temperature, ground and kept separately in airtight containers.

4.3. Extraction and isolation

Dried powdered root (300 g) was extracted at ambient temperature with 3 × 2.5 L acetone for one day each, and then with 3 × 2.5 L methanol for another three consecutive days. Both filtrates were collected separately and evaporated to dryness under reduced pressure. Each of the crude extracts was partitioned between petroleum ether (500 ml × 3) and 95% methanol: water (500 ml × 3). After evaporating each layer to dryness, the methanol fraction was subsequently partitioned between ethyl acetate (500 ml × 3) and water (500 ml × 3). The fractions RAW and RMW (water fractions from the original acetone and methanol extract respectively) were partitioned between 1-butanol (500 ml × 3), and water (500 ml × 3). The solvent / solvent partitions yielded eight crude fractions, RAP (0.65 g), RAE (1.00 g), RAB (0.055 g), RAW (0.04 g), RMP (0.005 g), RME (0.87 g), RMB (0.63 g), and RMW (4.55 g) which were evaporated to dryness and kept at 0–5 °C.

Part of the acetone-ethyl acetate crude fraction (RAE; 0.44 g) was chromatographed over 60 g silica gel by open column chromatography. The extract was eluted by 100% toluene (600 ml) and then 600 ml each of mixtures of toluene and ethyl acetate with increasing polarity, i.e. 1–10%, 20%, 30%, 50% ethyl acetate in toluene and finally 100% ethyl acetate. Fractions (40 ml) were collected separately, and monitored by TLC. Fractions showing the same Rf values were combined, and evaporated to dryness to yield the sub-fractions, RAE1-RAE17.

These subfractions were further separated by repeated silica gel chromatography using cyclohexane: chloroform and cyclohexane: ethyl acetate solvent gradients to yield ferruginol (4 mg), 7α-acetoxy-14-hydroxy-8,13-abietadiene-11,12-dione (7-*O*-acetylhorminone tautomer **5**; 8 mg) and 7α,14-dihydroxy-8,13-abietadiene-11,12-dione (horminone tautomer **6**; 5 mg), salvinolonyl-12-methyl ether (**7**) (8 mg) and two novel diterpenoids **1** (1-oxomicrostegiol; 6 mg), and **2** (viroxocin; 5 mg). β-sitosterol, oleanolic acid and ursolic acid were also isolated.

The acetone-petroleum ether crude fraction (RAP; 0.55 g) was similarly separated over 60 g silica gel 60 yield subfractions RAP1-RAP11. Further chromatographic separation of these subfractions yielded microstegiol (**4**) (7 mg), a novel purple diterpenoid **3** (viridoquinone; 6 mg), 1-docosyl ferulate (**8**) (5 mg), and a mixture of 2-(4'-alkoxyphenyl) ethyl alkanoates (**9**) (6 mg).

The methanol-ethyl acetate crude fraction (RME; 0.77 g) was chromatographed over 60 g silica gel 60 by open column chromatography. The column was first eluted by 100% chloroform 600 ml, and then 600 ml each of the mixture of chloroform and methanol with increasing polarity, i.e. 1–10%, 20%, 30%, 50% methanol in chloroform and finally 100% methanol. Fractions (40 ml) were collected separately and monitored by TLC using the mobile phase chloroform: methanol (9:1). Fractions showing the same Rf values were combined and evaporated to dryness.

Fraction 80–103 (5–6% methanol in chloroform) was further purified by semipreparative reversed-phase HPLC. A peak at retention time 6.7 min was eluted by 40% aqueous acetonitrile, and identified as 2'', 3''-di-*O*-acetyl martynoside (**17**) (7 mg).

Dried powdered aerial parts (300 g) was extracted using the same procedure as the roots. The solvent / solvent partitions yielded eight crude fractions, AP (3.52 g), AE (3.90 g), AB (0.87 g), AW (0.61 g), MP (0.52 g), ME (1.98 g), MB (3.19 g), and MW (11.69 g) which were evaporated to dryness and kept at 0–5 °C.

Part of the AE crude fraction (2.5 g) was separated on a column of silica gel 60 (100 g) eluted with 1 L of 100% toluene and followed by mixtures of toluene and ethyl acetate of increasing polarity containing from 1 to 10%, and then 20%, 30%, 40%, 50%, 80% of ethyl acetate in toluene and finally 100% ethyl acetate. Fractions (40 ml) were collected and evaporated to dryness and monitored by TLC using the mobile phase toluene: ethyl acetate (7:3). Fractions with similar Rf values were combined to give 14 subfractions (AE1–14).

These subfractions were further chromatographed over silica gel 60 using toluene: ethyl acetate, cyclohexane: ethyl acetate and chloroform: ethyl acetate solvent gradients to yield β-sitosterol (10 mg), two triterpenoid acetates **10** (6 mg) and **11** (5 mg), lup-(20)29-ene-2α, 3β-diol (**12**) (6 mg), oleanolic acid (10 mg) and ursolic acid (22 mg).

A further aliquot of AE crude fraction (1.4 g) was separated on silica gel 60 (100 g) eluted by 1 L of 100% chloroform, and then with increasing polarity by adding methanol from 1–10%, 20%, 30%, 50%, and finally 100% methanol to yield β-sitosterol glucoside (10 mg).

A solution of the polar fraction MB (2 g) in methanol was separated by semipreparative HPLC on a PFP column using methanol:

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water (45:55) as mobile phase. Sub-fractions and peaks were collected separately over the total retention time of 45 min, i.e. peaks of retention time 2.0–7.5 min, 7.5–13.5 min, 15.5 min, 16.7 min, 18.7 min, and 19–45 min. The sub-fraction from retention time 2.0–7.5 min was further chromatographed on the same column using 13% acetonitrile in water as mobile phase. Caffeic acid (14 mg), rosmarinic acid (3 mg), and 6-*O*-caffeoyl-glucose (**18**) (9 mg) were collected at retention times 6.0, 6.3, and 8.9 min respectively. The sub-fraction from retention time 7.5–13.5 min was further purified using the mobile phase 45% methanol in water. Salidroside (4 mg) was isolated as a peak at retention time 8.2 min. The major peak at retention time 9.2 min was identified as trans-verbascoside (**13**) (104 mg). A peak at retention time 11.2 min was identified as a mixture of trans-, and cis-verbascoside (**14**) 15 mg. Leucosceptoside A (**15**) (10 mg) was collected from a peak at retention time 15.5 min. The peak at retention time 16.7 min was identified as luteolin-7-rutinoside (9 mg) and luteolin-7-*O*-α-rhamnopyranosyl (1 → 6)-β-galactopyranoside (4 mg). The peak at retention time 18.7 min was luteolin-7-*O*-β-glucopyranoside (14 mg). The sub-fraction from retention time 19–45 min was further separated by 25% acetonitrile in water on the same column. Peaks at retention time 5.1, 7.9, and 9.5 min were identified as luteolin-7-*O*-β-galactopyranoside (5 mg), apigenin-7-*O*-β-glucopyranoside (6 mg), and martynoside (**16**) (6 mg) respectively.

4.4. 1-Oxomicrostegiol (compound **1**;10a-8,9,10,10a-tetrahydro-10a-hydroxy-6,10,10-trimethyl-2-(1-methylethyl)-cyclohepta[de]naphthalene-1,7-dione)

Compound **1** ($C_{20}H_{24}O_3$) was obtained as a brown-yellow powder. HR-ESI-MS (positive ion mode) showed a peak at *m/z* 335.1610; $C_{20}H_{24}O_3Na$ requires 335.1618. UV (MeOH): λ_{max} (log ε) = 312 (3.87), 278 (3.71), 253 (4.17), 236 (4.11). ^1H and ^{13}C NMR data are shown in Table 1.

4.5. Viroxocin (compound **2**; 3,4-Dihydro-11-hydroxy-10-(1-methylethyl)-2,2,6-trimethyl-naphtha[1, 8-bc]oxocin-5(2H)-one)

Compound **2** ($C_{20}H_{24}O_3$) was obtained as a brown-yellow powder. HR-ESI-MS (positive ion mode) showed a peak at *m/z* 335.1628; $C_{20}H_{24}O_3Na$ requires 335.1618 while the negative mode showed a peak at *m/z* 311.1642; $C_{20}H_{23}O_3$ requires 311.1653. UV (MeOH): λ_{max} (log ε) = 337 (3.56), 294 (3.72), 237 (4.45). ^1H and ^{13}C NMR data are shown in Table 1.

4.6. Viridoquinone (compound **3**; 5-Methyl-20-nor-abieta-1(10),6,8,13-tetraen-11,12-dione)

Compound **3** ($C_{20}H_{24}O_2$) was obtained as a purple powder. HR-ESI-MS (positive ion mode) showed a peak at *m/z* 319.1662; $C_{20}H_{24}O_2Na$ requires 319.1669. UV (MeOH): λ_{max} (log ε) = 460 (2.42), 325 (3.86), 285 (3.99), 246 (4.32). ^1H and ^{13}C NMR data are shown in Table 2.

4.7. 7α-Acetoxy-14-hydroxy-8,13-abietadiene-11,12-dione (**5**)(7-O-acetylhorminone tautomer)

Compound **5** ($C_{22}H_{30}O_5$) was obtained as a yellow powder. HR-ESI-MS (positive ion mode) showed a peak at *m/z* 397.1974; $C_{22}H_{30}O_5Na$ requires 397.1985 while the negative ion mode found at *m/z* 373.2004; $C_{22}H_{29}O_5$ requires 373.2020. ^1H NMR data (500 MHz, $CDCl_3$): δ 7.12 (1H, s, 14-OH), 5.92 (1H, dd, *J* = 4, 1.5 Hz, H-7-β), 3.15 (1H, septet, *J* = 7 Hz, H-15), 2.71(1H, ddd, *J* = 13, 4, 1 Hz, H-1-β), 2.03 (3H, s, -OCOC<u>H</u>$_3$), 1.92 (1H, br d, *J* = 15 Hz, H-6-α), 1.72 (1H, dddd, *J* = 13, 6, 2 Hz, H-2-α), 1.59 (1H, ddd, *J* = 15, 11, 1.5 Hz, H-6-β), 1.57 (1H, m, H-2-β), 1.49 (2H, br d, *J* = 13, 1 Hz, H-3-α and H-5-α), 1.22 (3H, s, H-20-β), 1.20 (3H, d, *J* = 7 Hz, H-16), 1.20 (2H, overlapped, H-1-α and H-3-β), 1.18 (3H, d, *J* = 7 Hz, H-17), 0.88 (3H, s, H-18-α), 0.87 (3H, s, H-19-β). ^{13}C NMR data (125 MHz, $CDCl_3$): δ 185.4 (C-11), 183.7 (C-12), 169.4 (-O<u>C</u>OCH$_3$), 150.7 (C-14), 149.9 (C-9), 139.4 (C-8), 124.6 (C-13), 64.5 (C-7), 46.1 (C-5), 41.0 (C-3), 39.0 (C-10), 35.8 (C-1), 32.9 (C-4 and C-18), 24.6 (C-6), 24.1 (C-15), 21.6 (C-19), 21.1 (-OCO<u>C</u>H$_3$), 19.8 (C-16), 19.7 (C-17) 18.8 (C-2), 18.5 (C-20).

4.8. 7α,14-Dihydroxy-8,13-abietadiene-11,12-dione (**6**)(horminone tautomer)

Compound **6** ($C_{20}H_{28}O_4$) was obtained as a yellow powder. HR-ESI-MS (positive ion mode) showed a peak at *m/z* 355.1880; $C_{20}H_{28}O_4Na$ requires 355.1880 while the negative ion mode showed a peak at *m/z* 331.1898; $C_{20}H_{27}O_4$ requires 331.1915. ^1H NMR data (500 MHz, $CDCl_3$): δ 7.20 (1H, s, 14-OH), 4.72 (1H, dd, *J* = 3.5, 2 Hz, H-7-β), 3.15 (1H, septet, *J* = 7 Hz, H-15), 3.00 (1H, s, 7-OH-α), 2.70 (1H, dt, *J* = 13.5, 2.5 Hz, H-1-β), 1.94 (1H, ddd, *J* = 15 Hz, H-6-α), 1.70 (1H, m, H-2), 1.60 (1H, overlapped, H-6-β), 1.53 (1H, m, H-2), 1.49 (1H, br d, *J* = 10.5 Hz, H-5-α), 1.46 (1H, m, H-3), 1.25 (1H, m, H-3), 1.22 (3H, s, H-20-β), 1.20 (6H, overlapped, H-16 and H-17), 1.18 (1H, m, H-1-α), 0.98 (3H, s, H-18-α), 0.90 (3H, s, H-19-β). ^{13}C NMR data (125 MHz, $CDCl_3$): δ 189.1 (C-12), 185.0 (C-11), 151.0 (C-14), 147.8 (C-9), 143.2 (C-8), 124.2 (C-13), 63.2 (C-7), 45.7 (C-5), 41.0 (C-3), 39.1 (C-10), 35.8 (C-1), 33.1 (C-18), 32.9 (C-4), 25.8 (C-6), 24.0 (C-15), 21.7 (C-19), 19.9 (C-16), 19.8 (C-17), 18.8 (C-2), 18.4 (C-20).

4.9. A mixture of 2-(4'-alkoxyphenyl) ethyl alkanoates (**9**)

A mixture of 2-(4'-alkoxyphenyl) ethyl alkanoate was obtained as a white powder. CIMS and GC-CI-MS were carried out using NH_3 as reagent gas at the EPSRC National Mass Spectrometry Service Center, School of Medicine, University of Swansea. The GC-CI-MS data (positive ion mode) showed three main components at *m/z* 478.4245; $C_{30}H_{52}O_3NH_4$ requires 478.4255, 506.4557; $C_{32}H_{56}O_3NH_4$ requires 506.4593 and 534.4869; $C_{34}H_{60}O_3NH_4$ requires 534.4881. ^1H NMR data (500 MHz, $CDCl_3$): δ 7.07 (2H, d, *J* = 8.5 Hz, H-2' and H-6'), 6.75 (2H, d, *J* = 8.5 Hz, H-3' and H-5'), 4.23 (2H, t, *J* = 7 Hz, H-1), 3.64 (2H, t, *J* = 6.5 Hz, H-α-alkoxy), 2.85 (2H, t, *J* = 7 Hz, H-2), 2.27 (2H, t, *J* = 7.5 Hz, H-β-alkanoate), 1.25–1.56 (-C<u>H</u>$_2$-), 0.88 (6H, t, *J* = 7 Hz, -C<u>H</u>$_3$ of alkoxy and alkanoate). ^{13}C NMR data (125 MHz, $CDCl_3$): δ 173.86 (C-α-alkanoate), 154.17 (C-4'), 130.04 (C-1', C-2' and C-6'), 115.29 (C-3' and C-5'), 64.90 (C-1), 63.12 (C-α-alkoxy), 34.34 (C-2), 34.26 (C-β-alkanoate), 32.81 (C-β-alkoxy), 22.69–29.46 (-<u>C</u>H$_2$-), 14.11 (<u>C</u>H$_3$ of alkoxy and alkanoate).

4.10. 2α-Acetoxy-lup-20(29)-en-3β-ol (**10**)

($C_{32}H_{52}O_3$) was obtained as a white powder. HR-ESI-MS (positive ion mode) showed a peak at *m/z* 507.3784; $C_{32}H_{52}O_3Na$ requires 507.3809. ^1H and ^{13}C NMR data are given in Table 3.

4.11. 3β-Acetoxy-lup-20(29)-en-2α-ol (**11**)

($C_{32}H_{52}O_3$) was obtained as a white powder. HR-ESI-MS (positive ion mode) showed a peak at *m/z* 507.3794; $C_{32}H_{52}O_3Na$ requires 507.3809 while negative ion mode found *m/z* 483.3462; $C_{32}H_{51}O_3$ requires 483.3480. ^1H and ^{13}C NMR data are given in Table 3.

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4.12. UVA irradiation of trans-verbascoside (trans → cis isomerisation)

The UV dose was measured using an IL1700 radiometer (International Light, Newbury, MA). Trans-verbascoside (10 mg) (98% trans by ^{1}H NMR) in CD_3OD (0.5 ml) was placed in a plastic plate and irradiated using UV radiation in the range of 350–400 nm at a dose of 500 kJ/m^2 by a Sellas 4 kW UVA lamp (Sellas, Germany). The ^{1}H NMR spectrum of the sample was re-recorded immediately after irradiation.

4.13. MIC assay for antibacterial activity

The panel of test organisms consisted of *S. aureus* (ATCC 6571), *E. faecalis* (ATCC 775), and *B. cereus* (ATCC 2599). Nutrient broth was supplied by Oxoid. *p*-Iodonitrotetrazolium chloride (INT) and chloramphenical were supplied by Sigma. All procedures were carried out under sterile conditions. A 96 well microplate technique for MIC assay was based on Eloff (1998) with modifications. Each bacterium species was seeded in the nutrient broth and grown for 24 h at 37 °C on a shaking bath. All compounds were dissolved in DMSO to make stock solutions of 20 mM, and further diluted with water to 200 μM for the highest test concentration. A series of two fold dilutions of each compound was prepared and 100 μl of each dilution was placed in a well in a 96 well plate. To each well, 100 μl cell suspension of the test bacterium (1×10^5 CFU/ml) was added, mixed and incubated for 24 h at 37 °C on a microplate shaker. The final concentration of DMSO was not more than 1% in each well. The toxicity of DMSO was also evaluated starting from 10% followed by two fold dilutions for six serial wells. To visualize the bacterial growth, 50 μl INT solution (2 mg/ml in water) was added into each well, mixed and further incubated for 30 min at 37 °C on a microplate shaker. The presence of bacteria was indicated by a purple color whereas a clear well was observed when bacterial growth was inhibited. The MIC was reported as the minimal concentration of compounds that inhibited the growth of bacteria. The nutrient broth was used as a blank, and the inoculated nutrient broth was the negative control. Chloramphenical (stock solution 200 mM in methanol) was employed as the positive control, diluted in water to 2 mM with further two fold dilutions for 12 serial wells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2014.08.029.

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